STUDIES ON NEURONAL APOPTOSIS FOLLOWING SOMAN EXPOSURE IN THE RAT

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ABSTRACT

Exposure to chemical warfare nerve agents (CWNA) is an ongoing threat to military personnel and civilian populations. The pathological consequences of CWNA exposure, including apoptosis, have not been well characterized. In the present study we have characterized the time course of the neuronal apoptotic events (caspase-3 activation, high molecular weight DNA fragmentation and 'Comet' assay analysis) occurring out to 48 h following Soman exposure. Male Sprague-Dawley rats were pre-treated with the oxime HI-6 (125 mg/kg, i.p.) and exposed 30 min later to 1.6x LD_{50} of Soman (180 μg/kg, s.c.) followed at 1 min by atropine (4 mg/kg, i.m.). Brains were removed and three brain regions (thalamus, hippocampus and piriform cortex) dissected at several post-exposure time points to evaluate parameters of apoptosis. A significant increase in caspase-3 activation was measured 2 h and 6 h post-exposure which was maximum at 2 h post-exposure. Highest activation of caspase-3 was observed in the thalamus followed by hippocampus and piriform cortex. Comet assay analysis showed significantly increased apoptosis and DNA fragmentation at 24 and 48 h post-exposure. Apoptosis was maximal in the thalamus at 24 h, followed by hippocampus and piriform cortex. In conclusion, an apoptotic cascade is activated following Soman exposure, which along with necrotic cell death may be involved in the neuropathology associated with Soman exposure.

INTRODUCTION

Approximately 700,000 military personnel were deployed to the Persian Gulf during 1990-91 for operation Desert Shield/Desert Storm. Although the rate of non-battle injuries during this conflict was very low relative to other such operations, there was a constant problem about the use of chemical warfare. Despite the fact that US military surveillance teams detected no exposures to chemical weapons, other countries claim to have detected low-level gaseous nerve agents (1). More recently, the potential for terrorists to use nerve agents became a reality when sarin gas was released in a Japanese subway in March of 1995. Such chemical terrorist attacks pose a definite threat to both civilians and military personnel in the US, and as such, research into methods of protecting against nerve-agent induced tissue and brain injury has become of paramount importance.

Classical nerve agents, such as soman, sarin, tabun and VX are extremely toxic organophosphates that can be used in military operations, or by terrorists, to kill, incapacitate, or seriously injure opponents. Organophosphates exert their effects by inactivating the enzyme acetylcholinestrase and causing accumulation of acetylcholine at neuronal synapses and at neuromuscular junctions, resulting in hyperactivity of the cholinergic system, and tetany of skeletal muscles, including the diaphragm (2). Traditionally, anticholinergic compounds have been demonstrated to provide variable degrees of neuroprotection against organophosphates (3). Recent studies have also attributed involvement of

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NMDA receptors modulation along with anticholinergic properties of neuroprotective drugs like caramiphen against OP toxicity (4). Neuronal apoptosis has been documented in acute injuries such as trauma (5), ischemia (6) and stroke (7,8), where activation of NMDA/glutamate receptor complex is documented as an early event. Cellular apoptosis and necrosis occur after similar tissue injuries (9,10) and specific morphological and biochemical features can distinguish these cytolytic mechanisms. For apoptosis, two well- studied markers include activation of caspase (an early event occurring within 2-4 hr after the insult) and "DNA laddering" (an event occurring 12-24 hr after the insult) (11,12). Histologically, apoptosis is characterized by preserved membrane integrity, condensation of cytoplasm and nucleus, diminished cellular volume, plasma bleb formation, and morphological preservation of organelle structure. Finally, in apoptosis, the cell fragments into apoptotic bodies that are engulfed by nearby cells and degraded (13). Alternatively, necrosis is distinguished by loss of membrane integrity, morphological indications of organelle damage, nuclear flocculation, loss of lysosomal contents, cellular swelling and uncontrolled cell lysis (14). Neuronal apoptosis in response to CNS injury is a well-studied phenomenon, but neuronal apoptosis in response to CWNA-mediated injury is less well studied. Similarly, although cellular apoptosis is a well-documented process following trauma, ischemia or stroke, its involvement in nerve agent-induced cellular degeneration remains to be elucidated. In this preliminary report, we quantify the level of neuronal apoptosis associated with soman-mediated injury using the single cell electrophoresis or "Comet" assay.

MATERIALS AND METHODS

Twenty-five male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) weighing 250-300g were maintained in animal rooms at 21 ± 2°C with 50 ±10% humidity. Food and water were provided before and after all procedures and animals were kept on a 12-h light/dark full spectrum lighting cycle with no twilight. Rats were pre-treated with the oxime HI-6 (1-2-hydroxy-iminomethyl-1-pyridino-3 (4-carbamoyl-1-pyridino-2-oxapropane dichloride), dissolved in sterile water, 125 mg/kg, i.p.). Thirty minutes later rats were exposed to a 1.6x LD_{50} dose of soman (pinacolyl methyl-phosphonofluoridate, 180 μg/kg, s.c.) followed at 1 min by treatment with atropine methyl nitrate dissolved in sterile water (4 mg/kg, i.m.). Vehicle treated animals were administered an identical treatment protocol except saline (s.c.) was substituted for soman. Following exposure brain was dissected and the hippocampus, thalamus, and piriform cortex removed at 2, 6, 24, or 48 h post-soman exposure for various analysis (4-5 rats per group). Tissue was macerated through a 100 μm nylon cell strainer (BD Falcon) into 10 mL ice cold 1 x phosphate buffered saline, calcium and magnesium free. The mixture was centrifuged at 1500g for ten minutes to remove cell debris. One hundred micro liters was removed and resuspended in 1mL of PBS for comet assay and 400 μL was removed and suspended in 1 x lysis buffer for Caspase-3 assay (see below).

Cells for comet analysis were washed twice by centrifugation for five minutes at 2000rpm. The final pellet was suspended in 1mL of PBS and 50 μL (1 x 10^5 /ml neurons) was combined with 500μL molten LM Agarose (Trevigen; Gaithersburg, MD) warmed to 42°C. Immediately, 50 μl was pipetted onto CometSlide (Trevigen, Gaithersburg, MD). Using the side of pipette tip agarose/cells were spread over sample area and placed at 4°C in the dark for 15 minutes. A 0.5 mm clear ring appears at inside edge of CometSlide area. Slides were immersed in prechilled Lysis Solution containing one percent DMSO (Trevigen) and left on ice for 30 minutes. After removal of excess buffer, slides were placed in a 50 ml Coplin jar containing 1X Alkali buffer (0.6 g NaOH pellets; 250 μl 200mM EDTA; 49.75 ml deionized water) for 30 min at room temperature in the dark. Slides were then washed twice in 1X TBE (Tris: Borate: EDTA) buffer for 3 minutes at room temperature in the dark. Slides were transferred from 1X TBE buffer and placed flat onto a gel tray submerged in 1X TBE buffer in a horizontal electrophoresis apparatus. The slides were aligned equidistant from the electrodes and electrophoresed for 10 minutes at

25V. Samples were fixed for five minutes in ice cold 100% methanol followed by ethanol, dried and stored.

To quantify DNA fragmentation, individual slides were covered with 50 µl of 0.01% SYBR Green (Trevigen) in Tris-EDTA buffer and imaged with a monochrome digital camera connected to an Olympus BX60 fluorescent microscope. Specially designed Comet Analysis software (Loats Associates, Westminster MD) was used to analyze the degree of DNA fragmentation in individual neurons. A direct count was taken to determine the amount of apoptosis. 500 cells were counted per animal and the percentage that was clearly apoptotic determined. Neurons were only scored as apoptotic it there was a clear comet tail and granularity of the fluorescent signal in the nucleus.

Caspase-3 activity was measured by a colorimetric method using a protocol and kit from Sigma Chemicals (St. Louis, MO). Briefly, the 400 µL cell suspension and lysis buffer was centrifuged at 3000 rpm for 10 minutes to remove cell debris and collect supernatant. The supernatant was removed and kept on ice. In a 96 well plate, 50 µl sample supernatant, 20 µl 5x assay buffer, 20 µl water and 10 µl Caspase substrate were combined. This was performed in triplicate for each sample. The plate was incubated at room temperature overnight in the dark. The plate was read at 405nm using a Ceres UV900 plate reader.

RESULTS

Figure 1 represents the percent of neurons exhibiting apoptosis after soman exposure at different time points in three regions of rat brain. Three to four rats were in each group, and 500 to 750 neurons were counted per animal. Only cells that exhibited a clear comet head and tail, as well as nuclear granularity, were counted as apoptotic. Error bars represent the standard error of the mean (n=3 to 4 per group). Neurons with DNA fragmentation were infrequent in saline-injected animals, but were significantly more numerous in all three brain regions in soman-injected animals. As shown in Figure 1, apoptosis increased with time and became maximal at 24-48 hours post soman exposure. The maximal effect was seen in the thalamus at 24 hours where 14% of neurons were apoptotic compared to just over 3% in the saline control group. Figure 2 shows representative comet images in both the groups in 3 brain regions.

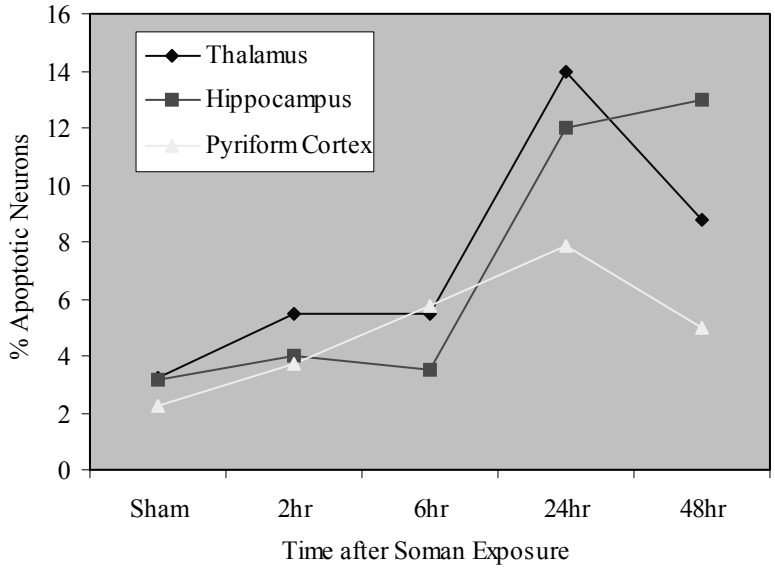

Figure 1. Percent of neurons exhibiting apoptosis after soman exposue at different time points in three regions of rat brain.

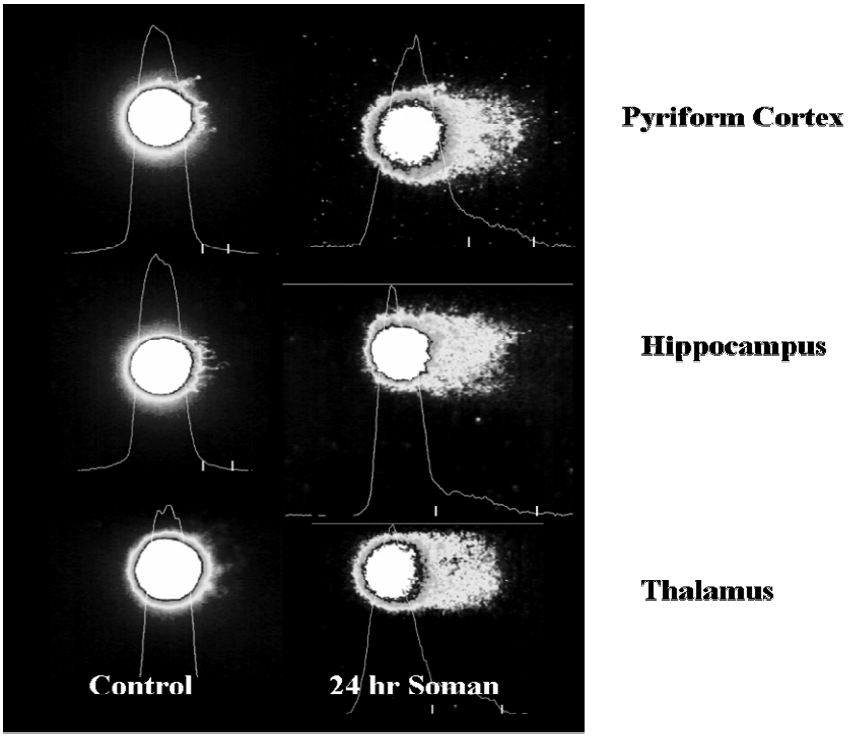

Figure 2. Representative images of neurons from different regions of rat brain from control (Left panels) and soman (Right panels) injected animals. The fluorescence intensity for each vertical strip of pixels in the image is summed and shown as an overlaid graph.

A significant increase in Caspase-3 activity was observed at 2 and 6 hours post soman exposure, which was maximal at 2 hours as shown in Figure 3. The highest activation of caspase-3 was in the thalamus followed by hippocampus and piriform cortex. An increase of just over 300% was measured in the thalamus at 2 hours.

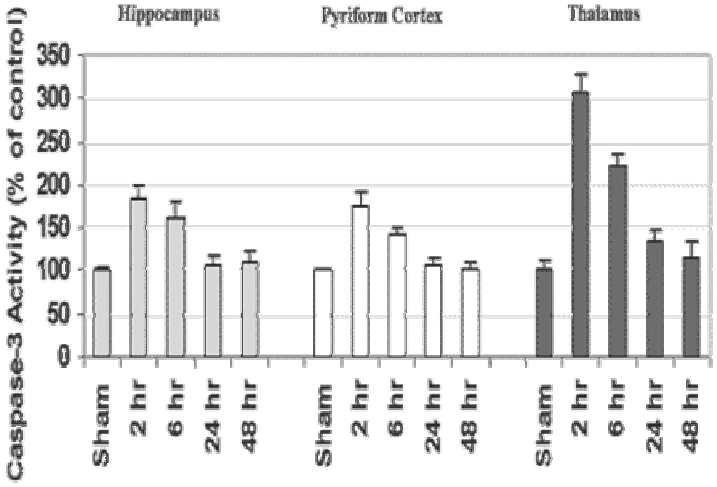

Figure 3. Caspase-3 activity in different regions of rat brain at different time intervals following soman exposure. Values are mean ± SEM, n=4-6.

CONCLUSION

Soman induced brain injury produced apoptosis across all brain regions studied. Morphologically, the percentage of neurons showing apoptosis increased with time and reached optimal levels by 24-48 hr post-soman exposures. Consistent with the apoptotic morphology were early increases in caspase-3 activity. Soman induced brain injury produced time-related increase in caspase-3 activation in three distinct brain regions. A maximal increase of approximately 300% in caspase-3 activity was observed in the thalamus. In conclusion, an apoptotic cascade is activated following Soman exposure, which along with necrotic cell death may be involved in the neuropathology associated with Soman exposure.

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FOOTNOTE

Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulation relating to animals and experiments involving animals and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, NIH publication 85-23. The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4-3), AR 360-5.